A Karyosystematic Study of the Genus *Bombina* from China (Amphibia: Discoglossidae)

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Abstract. -Chromosome number, morphology and positions of Ag-NORs were determined for four Chinese species of *Bombina*. Chromosome numbers are: *B. orientalis* (2n=24, NF=48), *B. maxima* (2n=28, NF=56), *B. microdeladigitora* (2n=28, NF=56). The Ag-NORs of *B. orientalis* are located on the long arm of the 7th chromosome pair, where as those of the latter three are on the short arm of the 11th pair. The subdivision of *Bombina* into two subgenera is supported by the karyology. A close relation between *Bombina* and *Discoglossus* is suggested.

Key words: Anura, Discoglossidae, *Bombina*; karyotypes, Ag-NORs, China.

TABLE 1. Species, localities, and number of individuals used in this karyological study.

Species	Locality	No. of Individuals	
B. (G.) fortinuptialis	Jinxiu, Guangxi	5 males	1 female
B. (G.) maxima	Dayao, Yunnan	11 males	5 females
B. (G.) microdeladigitora	Jingdon, Yunnan	5 males	2 females
B. (B.) orientalis	Qindao, Shandong	3 males	1 female

Introduction

There are five genera in the family Discoglossidae. The systematics of this family have long been under discussion. The systematic position of the genus *Bombina* within this family is the most problematical. A variety of studies dealing with this genus have been presented during the past years (summarized by Lang, 1988; 1989a; 1989b). However, the systematics of *Bombina* is still quite confusing. The relationships within *Bombina* have not been fully worked out.

Only six species belong to the genus *Bombina*. All are distributed in Eurasia. Karyological data are known for *B.bombina* (2n=24, NF=48), *B. variegata* (2n=24, NF=48) [Morescalchi, 1973], *B. orientalis* (2n=24, NF=48) [Jiang et al., 1984], *B. maxima* (2n=28, NF=56) [Zhao, 1986, no photographs presented]. No chromosome banding data are available for *Bombina*.

Careful morphological analysis and banding of the chromosomes may yield useful information not only on the phylogeny of the genus itself, but also on the relationships between *Bombina* and other genera of the family Discoglossidae. The purpose of this study is to analyze the karyotypes and Ag-NORs of four species in the genus from China Liu and Hu, 1961). The results, when compared with known karyological data of *B. bombina*, *B. variegata* and other genera of Discoglossidae, should be helpful in understanding the taxonomy and phylogeny of *Bombina*.

Materials and Methods

The specimens used in this investigation are listed in Table 1. The toads were collected by the authors at time of the year when both sexes are active for mating. The specimens were kept at room temperature (15-20°C) until the time of investigation.

To block mitosis at metaphase, we used a freshly prepared colchicine solution of 0.05%, and injected intraperitonealy 1/20ml of this solution per gram of body weight. The animals were sacrificed 20-24 hrs later, the spleen and small intestine were

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TABLE 2. Observational results of the diploid chromosome number of four species of *Bombina* from China.

Species	# of cells	Number of diploid chromosomes							
	observed	22	23	24	25	26	27	28	29
B. (G.) fortinuptialis	113					1	12	99	1
percentage (%)						0.88	10.6	87.6	0.88
B. (G.) maxima	198				3	3	14	176	2
percentage (%)					1.5	1.5	7	88.9	1
B. (G.) microdeladigitora	238				2	4	22	209	1
percentage (%)					0.8	1.7	9.2	87.8	0.4
B. (G.) orientalis	116	2	8	104	0	2			
percentage (%)		1.7	6.9	89.5		1.7			

removed, and the intestine was opened with a pair of fine scissors to expose the inner epithelial surface. The exposed epithelial surface was washed with a 0.64% NaCl solution for several minutes in order to remove all mucous and debris. The tissue was cut into small pieces and placed in a Petri-dish.

We added 8-10 ml of 0.4% KCl into the dish, suspended vigorously with a Pasteur pipette. The hypotonic treatment lasted 30-40 minutes. It was centrifuged at 1000 rpm for five minutes, and then the hypotonic solution was removed. The tissue was fixed with 8-10ml of freshly prepared solution of 3:1(v/v) absolute methanol-glacial acetic acid for three times, with a total time of 60 minutes. The samples will keep indefinitely in the fixative if stored at 1-4°C.

We prepared slides by transferring 3-4 pieces of the fixed tissue onto a dry, warm (about 50°C) slide. We then added 5-10 drops of 60% acetic acid and siphoned the solution up and down until the solution evaporated completely. Slides were stained in 10% Giemsa (pH 6.8) for 10 minutes. Staining of nucleolus organizer regions (NORs) followed the methods of Howell et al. (1980).

A total of 675 mitotic chromosome spreads were observed. Ten selected metaphase plates for each species were photographed, enlarged, and measured. The chromosome nomenclature used is that suggested by Levan et al. (1964). For the convenience of comparison, the chromosomes are defined as being large (A group), medium (B group) and small (C group) according to their relative lengths. Large chromosomes have a value of 100 units or more, small chromosomes have a value of 40 or fewer units. Chromosomes whose length falls between 40-99 units are considered to be medium.

Results

The observed diploid chromosome numbers are presented in Table 2. Measurements of metaphase chromosomes of four Chinese species of *Bombina* are shown in Table 3.

It is obvious that, the karyotypes of *maxima*, *microdeladigitora* and *fortinuptialis* are equal to each other. They have 2n=28, NF=56, composed of 6 pairs of large homologous, one pair of medium-small chromosomes and seven pairs of small homologous; all the chromosomes are metacentric (m), except for 6th, 7th, and 9th pair, which are submetacentric (sm). A weak secondary constriction is observed on the short arm of 11th pair. It appears in about 10% of the cases. The Ag-NORs are observed on short arm of 11th pair and

TABLE 3. Measurements of metaphase chromosomes of four Chinese species of *Bombina* Mean ± SE

B. fortinuptialis

Group	No.	Relative length	ratio	type
	1	160.0±4.22	1.18±0.06	m
	2	147.0±3.72	1.36±0.03	m
A	3	129.9±5.36	1.48±0.05	m
(1-6)	4	119.8±2.10	1.47±0.06	m
	5	109.9±5.20	1.21±0.08	m
	6	107.3±3.94	1.86±0.13	sm
B (7)	7	40.5±1.05	2.51±0.11	sm
	8	33.5±0.97	1.28±0.02	m
	9	28.4±0.69	2.19±0.09	sm
	10	27.4±1.27	1.50±0.03	m
C	11	26.4±0.78	1.48±0.05	m
(8-14)	12	25.4±0.76	1.62±0.08	m
	13	23.1±0.41	1.44±0.07	m
	14	21.4±0.71	1.22±0.04	m

B. maxima

Group	No.	Relative length	ratio	type
	1	165.2±5.30	1.23±0.10	m
	2	144.4±4.03	1.43±0.07	m
A	3	127.9±6.21	1.47±0.07	m
(1-6)	4	122.8±4.15	1.58±0.11	m
	5	108.1±6.52	1.38±0.11	m
	6	105.8±4.30	1.71±0.06	sm
B (7)	7	40.9±3.25	2.69±0.23	sm
	8	33.4±2.51	1.56±0.09	m
	9	30.1±1.75	2.33±0.12	sm
	10	28.1±1.02	1.37±0.06	m
C	11	26.7±1.33	1.42±0.08	m
(8-12	12	24.5±1.06	1.55±0.03	m
	13	22.8±1.10	1.44±0.02	m
	14	20.3±1.04	1.30±0.03	m

B. microdeladigitora

Group	No.	Relative length	ratio	type
	1	160.4±6.24	1.16±0.03	m
	2	142.8±5.87	1.45±0.10	m
A	3	130.1±5.56	1.54±0.08	m
(1-6)	4	120.5±3.78	1.52±0.07	m
	5	111.3±5.65	1.23±0.04	m
	6	104.6±4.19	1.90±0.13	sm
B (7)	7	41.8±2.96	2.56±0.16	sm
	8	34.7±1.01	1.39±0.07	m
	9	32.3±1.06	2.26±0.09	sm
	10	28.0±1.18	1.39±0.04	m
C	11	27.8±1.47	1.48±0.05	m
(8-14)	12	24.1±1.12	1.46±0.06	m
	13	22.1±2.04	1.44±0.08	m
	14	20.8±2.36	1.21±0.11	m

B. orientalis

Group	No.	Relative length	ratio	type
	1	152.3±4.89	1.14±0.02	m
	2	133.8±5.01	1.27±0.03	m
A	3	130.0±4.74	1.37±0.03	m
(1-6)	4	125.7±7.51	1.34±0.08	m
	5	113.0±2.30	1.22±0.11	m
	6	108.4±3.10	1.71±0.07	sm
B (7)	7	86.0±2.05	1.43±0.04	m
	8	38.4±3.25	1.07±0.03	m
	9	34.7±2.15	1.33±0.04	m
	10	28.7±1.76	1.42±0.06	m
C	11	26.1±1.75	1.15±0.13	m
(8-12)	12	24.1±1.04	1.53±0.05	m

relative length= (chromosome length/total of haploid chromosome length) x 1000
ratio= long arm/short arm

coincide with the position of secondary constriction. *B. orientalis* has 2n=24, NF≦48, consisting of 6 pairs of large homologues, one pair of medium-large chromosomes and 5 pairs of smaller homologues. All the chromosomes are m, except for the 6th pair, which is sm. A clear secondary constriction is found on the long arm of 6th pair, and a weak one is observed on the short arm of the 8th pair, the latter appears in a case of 5%. Ag-NORs were only observed on the long arm of 7th pair. No heteromophic chromosomes were found. The karyotypes

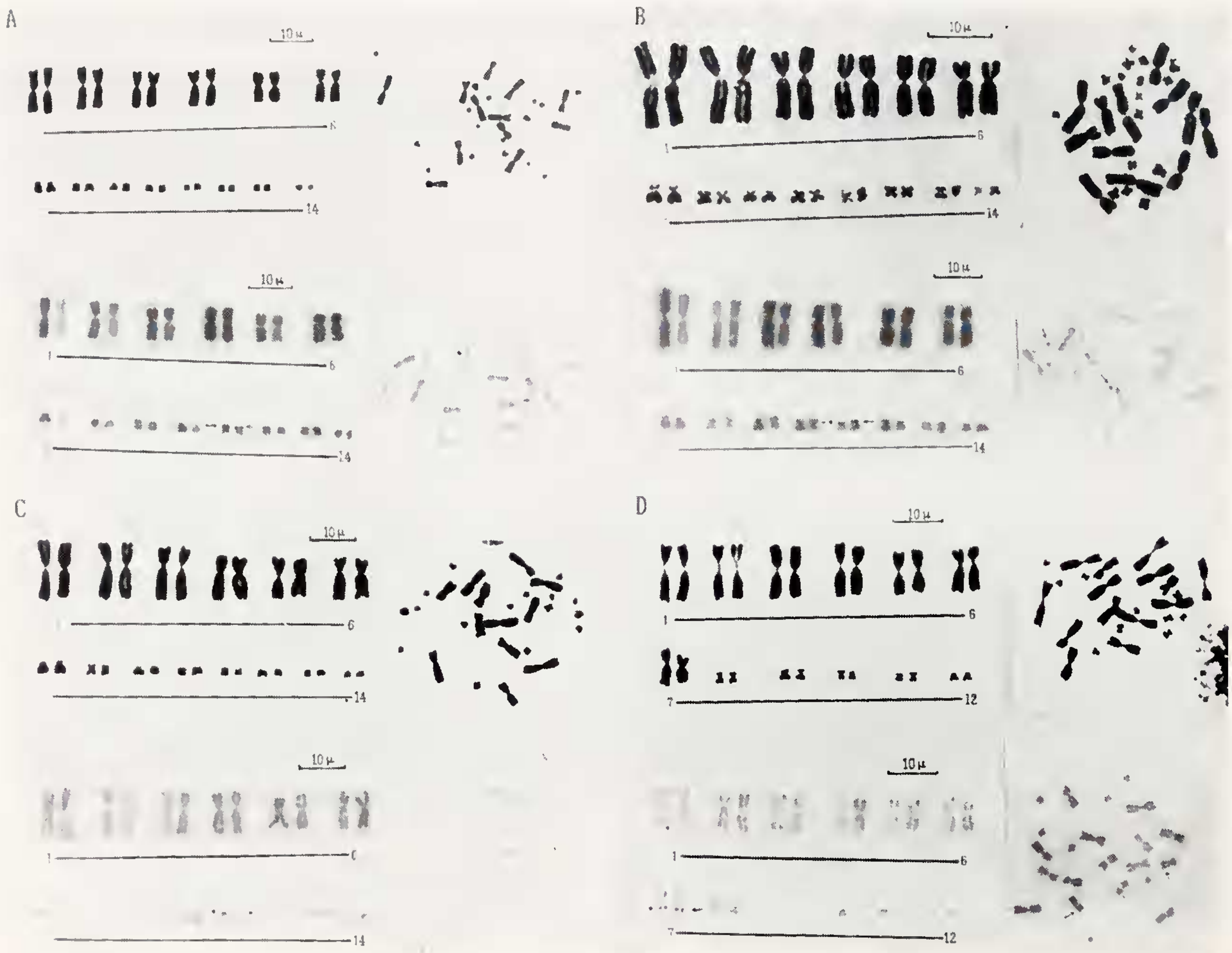

FIG. 1. Karyotypes and Ag NO3 stained karyotypes of *Bombina* from China. Arrows show Ag-NORs. A: *B. fortinuuptialis;* B: *B. maxima;* C: *B. microdeladigitora;* D: *B. orientalis.*

are presented in Fig. 1.

Discussion

Now, karyotypes are known for all the recognized species of *Bombina*. We compare the karyotypes and Ag-NORs of them in Table 4.

All the chromosomes of *Bombina* have median or submedian centromeres. In the discoglossids, *Alytes* are rich in acrocentrics, and with some microchromosomes (2n=38, NF=64-72), *Discoglossus* have 2n=28, NF=54, with one pair of telocentrics (Morescalchi, 1973). So, from the karyololgical point of view, *Bombina* is the most highly differentiated.

Within *Bombina*, two different kinds of karyotypes exist. The differences between the two are mainly as follows: 1). The morphology of 7th pair are quite different. The 7th pair of *maxima, microdeladigitora* and *fortinuptialis* are medium-small and s, where as those of *bombina, variegata* and *orientalis* are medium-large, m, with a clear secondary constriction on the long arm. 2). The number of smaller homologues is different. The former three have 7 pairs of small homologues, but the latter three have only 5 small homologues. Tian and Hu (1985) suggested a subdivision of *Bombina* into two subgenera, the subgenus *Bombina* containing the Palaearctic *bombina, variegata* and *orientalis*, and the Oriental *Glandula* containing *maxima*, *microdeladigitora* and *fortinuptialis*. Our

TABLE 4. Comparison of karyotypes and Ag-NORs in *Bombina*.

Species	2n	NF	Chromosome formula	Secondary centremere	Locality of A-NORs	Data
B. (G.) fortinuptialis	28	56	22m+6sm	No. 11	No. 11	Present study
B. (G.) maxima	28	56	22m+6sm	No. 11	No. 11	Present study
B. (G.) microdeladigitora	28	56	22m+6sm	No. 11	No. 11	Present study
B. (G.) orientalis	24	48	22m+2sm	Nos. 7,8	No. 7	Present study
B. (G.) bombina	24	48	unknown	Nos. 7,8	unknown	Morescalchi 1973
B. (G.) variegata	24	48	unknown	Nos. 7,8	unknown	Morescalchi 1973

karyological evidence support this subdivision.

In *B. maxima, microdeladigitora* and *fortinuptialis,* the karyotypes are practically equal to each other which indicates that these three species may have diverged recently. In the group consisting of *B. bombina, variegata* and *orientalis, bombina* and *variegata* are equal to one another (Morescalchi, 1973), but *orientalis* has some differences from them. The 8th and 12th pairs are m in *orientalis*, but st in *bombina* and *variegata*. Thus the two European species are more closely related, which is congruent with immunological evidence of Maxon (1979) and Maxson and Szymura (1984).

Morescalchi et al. (1977) could not resolve relationships within Discoglossidae because the karyotypes of *Discoglossus*, *Alytes* and *Bombina* are so different from each other. Our investigation indicated that *Discoglossus* and the *Glandula*-group of *Bombina* have the same diploid chromosome number (Lanza et al., 1975; 1976). We suggest that those two genera may be related. The secondary constriction is the only "marker" currently available for analysis in most anuran karyosystematics studies. However, in the present study, we found that the secondary constriction is abrupt and the position of it is quite variable. The Ag-NORs are stable and clear and may be a more useful tool than the place of the secondary constriction in some cases. The mechanisms of karyotype evolution of *Bombina* may take place through unequal translocation. This is still an open question. A further chromosome banding study is necessary.

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